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Food-Grade Cloning and Expression System for *Lactococcus lactis*

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A versatile set of cloning and expression vectors has been developed for application in self-cloning and other genetic modifications of *Lactococcus lactis*. The expression vectors were equipped with the controlled and strong *lacA* promoter of the lactococcal lactose operon. In addition, the transcriptional terminator of the aminopeptidase N gene, *pepN*, was inserted, which in some cases increased the genetic stabilities of the vectors and the cloned DNA. The small, 0.3-kb *lacF* gene encoding the soluble carrier enzyme IIA^{Lac} was used as a dominant selection marker in the plasmid-free *L. lactis* strain NZ3000 carrying an in-frame deletion of the chromosomal *lacF* gene. Lactose-utilizing transformants were easily selected on lactose indicator plates at high frequencies and showed a copy number of approximately 50 plasmids per cell. All vectors were stably maintained in the *lacF* strain NZ3000 when grown on lactose, and only the high-level expression vectors showed some instability when their host was grown on glucose-containing medium. The application potentials of the expression vectors carrying the *lacF* marker were determined by cloning of the promoterless *Escherichia coli gusA* reporter gene under control of the *lacA* promoter followed by analysis of its expression. While in one of the vectors this resulted in a promoter-down mutation in the −10 region of the *lacA* promoter, in other vectors high-level and controlled expression of the *gusA* gene was observed.

The development of a wide variety of cloning systems has allowed the improvement of many properties of *Lactococcus lactis* strains that are essential for a large number of industrial dairy and other food fermentations (10, 15). Those genetically improved lactococci and their products have great potential to be used in the food industry. However, the production strains used should be devoid of any antibiotic resistance markers that could compromise their applications in foods. As a consequence, vectors should contain selection markers that are acceptable in the food industry, and these are described here as food grade. Various food-grade systems have previously been proposed for *Lactococcus* spp. Some of these were based on homologous marker genes, such as the nisin resistance determinant *nsr* (14), while a heterologous system based on the sucrose utilization genes of *Pediococcus pentosaceus* has been designed (16). However, the application of these systems is limited to the cloning of a lactococcal bacteriophage resistance gene by using the *nsr* marker gene (14). The complementation of auxotrophic mutants is another approach to develop homologous markers that may allow for simple and dominant selection. Such a complementation system, based on nonsense suppressors of mutations in the lactococcal purine biosynthetic pathway, has recently been developed (12), while a marker system based on the *L. lactis* thymidylate synthase gene, *thyA*, has been proposed but not evaluated because of a lack of the appropriate mutants (19). The detailed characterization of the *L. lactis lac* operon encoding the lactose phosphotransferase system and tagatose-6-phosphate pathway (7, 8, 25, 26) has provided the possibility of developing a dominant homologous marker based on lactose complementation (6). The two elements of this system included the small, 0.3-kb *lacF* gene coding for the soluble enzyme IIA^{Lac}, which was expressed by a vector-located promoter, and the lactose-deficient *L. lactis* strain YP2-5, which contained a missense mutation in the *lacF* gene (7). Evaluation of this first example of a homologous selection system for lactic acid bacteria showed its advantages, which included simple selection with lactose indicator plates and high stability during growth on lactose-containing industrial media (6). However, in order to fully exploit this marker system, there has been a need to obtain a series of useful cloning and expression vectors. Here we describe the development and application of stable and versatile vectors based on the high-copy-number endogenous *L. lactis* pSH71 replicon, the *lacF* selection marker, and the lactose-inducible *lacA* promoter.

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MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The *L. lactis* strains and plasmids used in this study are listed in Table 1. *Escherichia coli* MC1061 was grown in Luria broth (20) at 37°C. *Lactococcus* strains were routinely grown at 30°C in M17 broth (Difco Laboratories, Detroit, Mich.) (23) supplemented with 0.5% lactose or glucose. The ability to ferment lactose was tested on indicator agar based on Elliker broth (13) containing 0.004% bromocresol purple and 0.5% lactose. Histochemical screening for *gusA*-positive clones was performed with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) (Research Organics Inc., Cleveland, Ohio) at a final concentration of 0.5 mM. Chloramphenicol (10 μg/ml) and ampicillin (100 μg/ml) were used when appropriate.

Molecular cloning, reagents, and enzymes. Plasmid DNA was isolated from *E. coli* by using the alkaline lysis method (1). Plasmid DNA from *L. lactis* was isolated by a modification of this procedure (8), and total DNA of *L. lactis* was isolated as described previously (16). Plasmid DNA was transformed into *L. lactis* by the method of Wells et al. (28). All other cloning procedures and *E. coli* manipulations were performed as described previously (20). Enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), New England BioLabs Inc. (Beverly, Mass.), or Boerhinger GmbH (Mannheim, Germany) and used according to the instructions of the manufacturers. Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.). *para*-Nitrophenyl-β-D-glucuronic acid was obtained from Clonetech Lab. Inc. (Palo Alto, Calif.).

The assay of β-glucuronidase activity to determine the promoter strength in the expression vectors with the *gusA* gene was as previously described (18).

TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant features	Reference or source
L. lactis MG5267	Lac$^+$, plasmid free, single chromosomal copy of *lac* operon	24
L. lactis NZ3000	Δ*lacF*, derived from MG5267 by replacement recombination	11
E. coli MC1061	*araD139 lacX74 galU galK hsr hsm$^+$ strA*	3
pUC19	Ap^r; cloning vector	27
pUC-T	Ap^r, T_{pepN}; 0.3-kb *Mlu*I-*Hin*dIII *pepN* terminator fragment joined to pUC19 *Hin*dIII-*Sal*I	This work
pNZ124	Cm^r; pSH71-derived lactococcal cloning vector	5, 18
pNZ272	Cm^r; *gusA* promoter-probe vector derived from pNZ124	18
pNZ1125	*lacF*; contains *pepN* gene	10
pNZ3004	Cm^r; pGKV210 derivative harboring the *lacA* promoter	24
pNZ2101	Cm^r, T_{pepN}; 0.3-kb *Hin*dIII-*Sal*I *pepN* terminator fragment from pUC-T inserted into *Xho*I-*Sal*I-digested pNZ124	This work
pNZ2102	Cm^r, P_{lacA}; 0.5-kb *Eco*RI-*Pst*I *lacA* promoter fragment of pNZ3004 inserted into *Pvu*II-*Pst*I-digested pNZ124	This work
pNZ2103	Cm^r, P_{lacA}, T_{pepN}; 0.5-kb *Eco*RI-*Pst*I *lacA* promoter fragment of pNZ3004 inserted into *Pvu*II-*Pst*I-digested pNZ2101	This work
pNZ2104	*lacF*; 0.4-kb *Nco*I-*Bam*HI *lacF* fragment of pNZ1125 inserted into *Sal*I-*Bgl*II-digested pNZ124	This work (Fig. 2)
pNZ2105	*lacF*, T_{pepN}; 0.4-kb *Nco*I-*Bam*HI *lacF* fragment of pNZ1125 inserted into *Sal*I-*Bgl*II-digested pNZ2101	This work (Fig. 2)
pNZ2106	*lacF*, P_{lacA}; 0.4-kb *Nco*I-*Bam*HI *lacF* fragment of pNZ1125 inserted into *Sal*I-*Bgl*II-digested pNZ2102	This work
pNZ2107	*lacF*, P_{lacA}, T_{pepN}; 0.4-kb *Nco*I-*Bam*HI *lacF* fragment of pNZ1125 inserted into *Sal*I-*Bgl*II-digested pNZ2103	This work
pNZ2120/pNZ2121	*lacF*, P_{lacA}; pNZ2106 derivative with a multiple cloning site	This work (Fig. 2)
pNZ2122/pNZ2123	*lacF*, P_{lacA}, T_{pepN}; 0.3-kb *Sst*I-*Nde*I *pepN* terminator fragment of pNZ2101 inserted into pNZ2120 or pNZ2121	This work (Fig. 2)
pNZ2116	*lacF*, P_{lacA}, T_{pepN}, *gusA*; 1.2-kb *Pst*I-*Hin*dIII *gusA* fragment of pNZ272 inserted into pNZ2102	This work
pNZ2118	*lacF*, P_{lacA}, T_{pepN}, *gusA*; 1.2-kb *Pst*I-*Hin*dIII *gusA* fragment of pNZ272 inserted into pNZ2106	This work
pNZ2119	*lacF*, P_{lacA}, T_{pepN}, *gusA*; 1.2-kb *Pst*I-*Hin*dIII *gusA* fragment of pNZ272 inserted into pNZ2107	This work

Vector constructions. The relevant properties of the contructed vectors are listed in Table 1. pUC-T was constructed by cloning the aminopeptidase N gene (*pepN*) terminator (22) as a 0.3-kb *Mlu*I (made blunt with Klenow DNA polymerase)-*Hin*dIII fragment in pUC19 (digested with *Hin*dIII and *Sal*I, with the latter made blunt with Klenow polymerase), using *E. coli* MC1061 as a host. The *pepN* terminator fragment was then isolated as a *Sal*I-*Hin*dIII fragment from pUC-T and cloned in the pSH71-based vector pNZ124 (18), which was digested with *Xho*I and *Hin*dIII. The resulting vector was designated pNZ2101. For the construction of vectors with high-level and controlled expression, the *L. lactis lacA* promoter was used to initiate transcription. The *lacA* promoter was isolated from plasmid pNZ3004 (24) digested with *Pst*I and *Eco*RI. The *Eco*RI site was made blunt with Klenow polymerase. The 0.5-kb fragment was cloned in vectors pNZ124 and pNZ2101 digested with *Pvu*II and *Pst*I, resulting in pNZ2102 and pNZ2103, respectively. Vectors pNZ2101, pNZ2102, and pNZ2103 were constructed in *L. lactis* MG5267.

For the construction of homologous vectors from pNZ2101, pNZ2102, and pNZ2103, the chloramphenicol acetyltransferase gene was deleted by a restriction digestion with *Sal*I (made blunt with Klenow polymerase) and *Bgl*II and replaced with the *lacF* gene, isolated as a 0.4-kb *Nco*I-*Bam*HI fragment from plasmid pNZ1125 (the *Nco*I site was made blunt). Nonessential restriction sites were removed from plasmid pNZ2106, and a polylinker with multiple cloning sites was cloned upstream of the *lacA* promoter. Vectors pNZ2120 and pNZ2121 were constructed from plasmid pNZ2106 in four steps: (i) the small *Xba*I fragment was deleted; (ii) the resulting plasmid was digested with *Sal*I, made blunt with Klenow polymerase, and religated to remove this site; (iii) the restriction sites upstream of the *lacA* promoter were removed by an *Eco*RI-*Kpn*I digestion, the ends were filled in with Klenow polymerase, and the vector was religated; and (iv) finally, a synthetic double-stranded linker (5′-CTAGACAGCTGGGATC CGTCGACCCCGGGCTGCAGGCATGCGGTACCACTAGTT-3′ and 5′-CT AGAACTAGTGGTACCGCATGCCTGCAGCCCGGGGTCGACGGATCCC AGCTGT-3′) was cloned in the unique *Xba*I site, resulting in a multiple cloning site with unique restriction sites for *Pvu*II, *Bam*HI, *Sal*I, *Sma*I, *Pst*I, *Sph*I, *Kpn*I, and *Spe*I. Vectors pNZ2122 and pNZ2123, which are derivatives of pNZ2107, were constructed by cloning the *Sst*I-*Nde*I fragment of pNZ2101 in the vectors pNZ2120 and pNZ2121 digested with the same enzymes. All of these vectors were constructed in *L. lactis* NZ3000.

The structures of the new plasmids that contained either the chloramphenicol resistance gene or the *lacF* gene as marker are summarized in Fig. 1 and were verified by single and double restriction enzyme digestions, while the orientations and sequences of the polylinkers carrying the multiple cloning sites were verified by nucleotide sequence analysis.

To test the application potentials of the vectors pNZ2102, pNZ2106, and pNZ2107, they were digested by *Pst*I and *Hin*dIII and used to clone the *E. coli* *gusA* gene, which was isolated from pNZ272 as a *Pst*I-*Hin*dIII fragment (18). *L. lactis* NZ3000 or the isogenic strain MG5267 was used as the host with ligation mixtures from either pNZ2106 and pNZ2107 or pNZ2102, respectively.

Determination of plasmid copy number per chromosome. Total DNA was digested with *Xho*I, separated by agarose gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized with a [γ-^{32}P]dATP-end-labeled *lacF* probe (5′-TTAATTACTTTGCTCCTCTGATCAC-3′). The hybridizing DNA fragments corresponding to the chromosomal and plasmid-encoded *lacF* genes were isolated, and the radioactivity was determined by using a liquid scintillation counter (LS7500; Beckman Instruments Inc., Palo Alto, Calif.). The ratio between the radioactivity obtained from the plasmid-located *lacF* gene and that obtained from the single chromosomal copy of *lacF* defines the copy number.

Nucleotide sequence analysis. The nucleotide sequences of the polylinkers in pNZ2110 and pNZ2111 and the *lacA* promoters in vectors pNZ2116, pNZ2118, and pNZ2119 were determined by the dideoxy-chain termination method (21), as modified by the AutoRead sequencing kit, and performed on the A.L.F. apparatus (Pharmacia Biotech, United Kingdom). A fluorescent primer specific for the *gusA* gene with the sequence 5′-GGGTTGGGGTTTCTACAGGACGTA-3′ was used (18).

RESULTS

Construction and characterization of food-grade cloning and expression vectors. To allow for the development of vectors carrying the *lacF* marker, a series of plasmids based on the lactococcal vector pNZ124 (18) was constructed; the plasmids have a common core structure consisting of the *L. lactis* promiscuous pSH71 replicon, the chloramphenicol resistance gene from the staphylococcal plasmid pC194, and a multiple cloning site (Fig. 1). Plasmids pNZ2102 and pNZ2103 were equipped with the controllable *L. lactis lacA* promoter (7, 24), while pNZ2101 and pNZ2103 additionally contain the *L. lactis pepN* terminator (22) (Table 1). These vectors were designed in such a way that a simple replacement cloning step would remove the chloramphenicol acetyltransferase gene and position the promoterless lactococcal *lacF* gene under control of the *repC* promoter (10) of the pSH71 replicon (Fig. 1). This resulted in the cloning vectors pNZ2104 and pNZ2105 (Fig. 2)

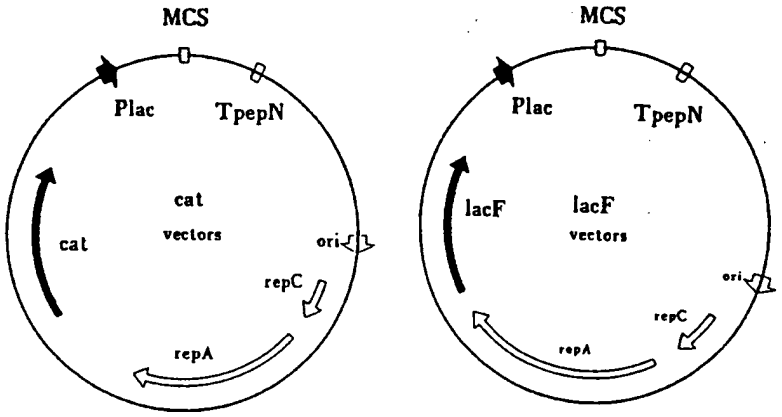

FIG. 1. Basic architecture of the constructed vectors carrying either a chloramphenicol resistance gene (left) or the *lacF* gene (right), which allows for food-grade selection. The minus origin of pSH71 (ori) is indicated, and the direction of the rolling-circle replication is indicated by the open arrow, which also indicates the direction of the *repC* promoter preceding the *repC* (repressor) and *repA* (replication protein) genes (10). MCS, multiple cloning site.

and the expression vectors pNZ2106 and pNZ2107, which harbor the *lacA* promoter without or with the *pepN* terminator, respectively. The two expression vectors were further improved by replacing nonessential restriction sites by both orientations of a newly designed polylinker, resulting in the pNZ2120/pNZ2121 and pNZ2122/pNZ2123, all of which contain the *lacA* promoter in the same orientation as the *repC* promoter driving expression of the *lacF* gene (Fig. 2). The lactose-inducible *lacA* promoter is known to induce high-level expression of downstream cloned genes (24). The construction of these vectors was performed in *L. lactis* NZ3000 by direct selection for acid formation from lactose on lactose indicator agar. All of the vectors carrying the *lacF* gene could easily be selected by lactose complementation of *L. lactis* NZ3000, indicating that the *repC* promoter drives efficient expression of the *lacF* gene. This was also evident from the rapid growth to high densities on lactose-containing medium of *L. lactis* NZ3000 harboring either one of the vectors (data not shown).

Application of the new vectors. To evaluate the use of the new vectors in gene cloning and controlled expression, the promoterless *E. coli* β-glucuronidase (*gusA*) gene was cloned under control of the *lacA* promoter. A plasmid with the expected size and configuration as determined by restriction enzyme digestion was readily obtained with pNZ2102, and the presence of this plasmid, designated pNZ2116, in *L. lactis* MG5276 gave rise to blue colonies on X-Gluc plates. However, when the *gusA* gene was inserted under control of the *lacA* promoter in pNZ2103, which also contained the *pepN* terminator, unexpectedly only white transformants of MG5276 were obtained on X-Gluc plates. Plasmid DNAs were isolated from 10 transformants, and their restriction digestion patterns showed a similar-sized plasmid that was larger than expected and hence not used for further studies.

The *gusA* gene was subsequently inserted into the expression vectors pNZ2106 and pNZ2107 and transformed into the *lacF*-deficient strain NZ3000. Lactose-utilizing transformants were readily obtained with plasmid pNZ2107 and gave rise to blue colonies on plates containing X-Gluc. All transformants tested contained a plasmid with the expected configuration, and one of those was designated pNZ2119. However, cloning of the *gusA* gene in pNZ2106 resulted in only one blue colony out of 500 transformants. The plasmid of this blue transformant showed the expected configuration and was designated pNZ2118.

Control of *gusA* gene expression in the *lacA* expression plasmids. To study the regulation of the *lacA* promoter in the different *gusA*-containing plasmids, the β-glucuronidase activities in lysates of cells grown in glucose or lactose were compared (Table 2). Strain NZ3000 harboring pNZ2118 showed the lowest β-glucuronidase activity and demonstrated no regulation (see below). The highest β-glucuronidase activity was observed in *L. lactis* NZ3000 harboring the vector pNZ2119. In this strain the induction of *gusA* expression upon growth on lactose-containing medium is twofold higher than that on glucose, as was also the case for strain MG5276 harboring pNZ2116.

Copy number and stability determination. The copy numbers of the new cloning and expression vectors in strain NZ3000 were compared (Fig. 3). Vectors pNZ2105, pNZ2106, and pNZ2107 have approximately the same copy number, whereas plasmids pNZ2104 and pNZ2118 (overexpressing the *gusA* gene), remarkably, showed a higher copy number. In contrast, plasmid pNZ2119, which also contains the *gusA* gene, has a copy number in *L. lactis* that is considerably reduced compared with those of the other plasmids. The exact plasmid copy numbers were determined for the expression vectors pNZ2106 and pNZ2107 and were found to be 46 and 36 copies per chromosome, respectively. Although no efforts to specifically detect insertion of the *lacF*-carrying plasmids in the chromosomal *lacF* locus were made, no such integration was ever observed in the Southern blot analysis during these copy number determinations.

The segregational instabilities of the vectors with or without the *gusA* gene were examined under selective and nonselective conditions. This was tested by growing strain NZ3000 harboring one of the plasmids in M17 medium containing 0.5% glucose or lactose and then plating appropriate dilutions on lactose indicator plates. All lactose-utilizing colonies appeared to have retained the plasmid. All of the vectors were stably maintained for more than 100 generations on lactose-containing M17 medium, and single colonies obtained after this period of growth were found to harbor plasmids with the expected size and restriction pattern (data not shown). Most of the vectors were also stably maintained when cultured on M17 medium

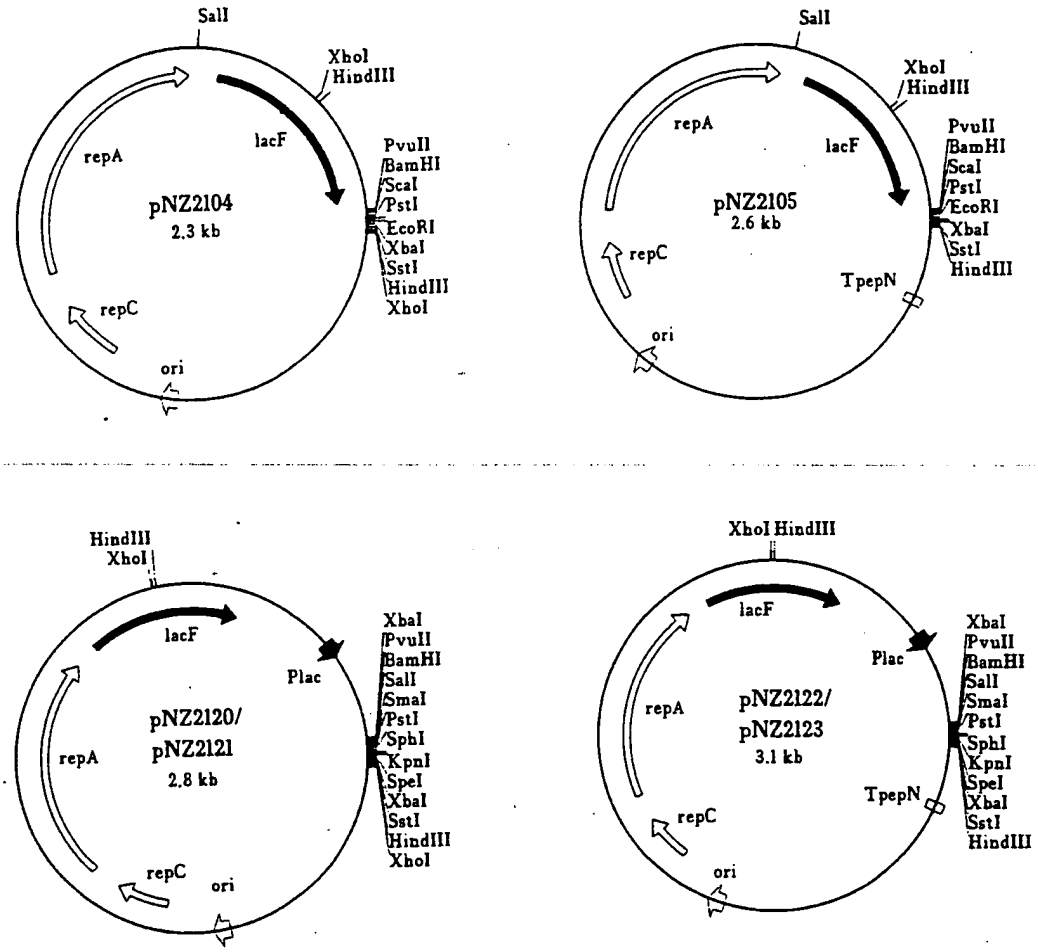

FIG. 2. Food-grade cloning and expression vectors. For explanations of symbols, see the legend to Fig. 1.

containing glucose (Table 3). However, when grown on glucose-M17, *L. lactis* NZ3000 harboring pNZ2118 or pNZ2106 showed a slightly reduced stability, while NZ3000 cells harboring pNZ2119 showed the highest segregational instability under these conditions.

Plasmid pNZ2118 contains a regulatory, promoter-down mutation in the *lacA* −10 region. To determine the origin of the variation in β-glucuronidase expression and to explain the observation that expression is not regulated in strain NZ3000

TABLE 2. β-Glucuronidase activities

Vector	β-Glucuronidase activity[a] (mean ± SD) of cells grown on:	
	Lactose	Glucose
pNZ2116	1,006 ± 128	586 ± 101
pNZ2118	367 ± 102	412 ± 137
pNZ2119	3,933 ± 486	2,043 ± 165

[a] Expressed as nanomoles per minute per milligram of protein. Data are for three independent determinations.

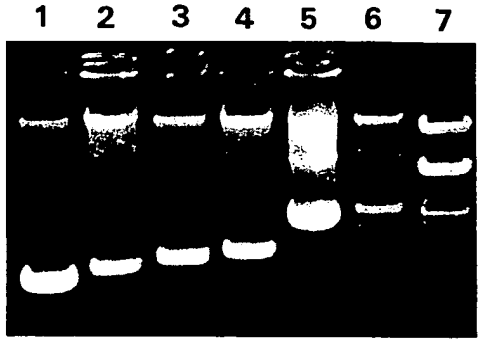

FIG. 3. Plasmid copy numbers of the food-grade cloning and expression vectors. Plasmid DNAs were extracted from equal amounts (based on the optical density of the culture) of NZ3000 cells harboring the different plasmids, digested with *Eco*RI, and separated by agarose gel electrophoresis. Lane 1, pNZ2104; lane 2, pNZ2105; lane 3, pNZ2106; lane 4, pNZ2107; lane 5, pNZ2118; lane 6, pNZ2119; lane 7, bacteriophage λ DNA digested with *Pst*I (marker).

TABLE 3. Stabilities of the food-grade vectors

Plasmid	Plasmid loss/generation
pNZ2104	6×10^{-4}
pNZ2105	1×10^{-4}
pNZ2106	2×10^{-3}
pNZ2107	9×10^{-4}
pNZ2118	3×10^{-3}
pNZ2119	1×10^{-2}

harboring pNZ2118, the nucleotide sequences of the *lacA* promoter in the *gusA*-containing plasmids pNZ2116, pNZ2118, and pNZ2119 were determined. Both pNZ2116 and pNZ2119 contained the bona fide *lacA* promoter (24). In contrast, the *lacA* promoter in plasmid pNZ2118 contained a single mutation at position −11, where a T residue was replaced by a C residue (Fig. 4).

DISCUSSION

To allow for further exploitation of the food-grade marker system based on the lactococcal *lacF* complementation system (7), we have constructed a variety of vectors that contained the *lacF* gene as a selective marker under control of the replicon promoter of the lactococcal plasmid pSH71. Introduction of these homologous vectors into *L. lactis* NZ3000, which contains an in-frame deletion in the *lacF* gene, resulted in rapid growth and acid formation on lactose-containing medium, allowing for simple and efficient selection as well as stable maintenance of transformants.

The utility of the new series of cloning vectors carrying the *lacF* marker was shown by their transformation into expression plasmids that were equipped with the *lacA* promoter, which is known to be involved in efficient and moderately controlled transcription initiation in *L. lactis* (24). Moreover, in some expression vectors the transcriptional terminator of the lactococcal *pepN* gene was inserted to prevent transcription into the pSH71 replication region, which in some cases resulted in increased stabilities of the vector and the cloned DNA, as also observed for streptococcal DNA in *E. coli* (4). Finally, the expression vectors were equipped with a polylinker containing multiple unique cloning sites, allowing easy and efficient cloning.

All vectors carrying the *lacF* marker showed high segregational stability in *L. lactis* NZ3000 when grown on lactose-containing medium, as a consequence of the nature of the complementation system. This allows for their effective use in industrial applications, because many food fermentations are based on whey-derived media that contain lactose as the sole energy source. In addition, no apparent physical alterations were observed in the vectors even after prolonged growth on lactose-containing medium, indicating that chromosomal integration is an infrequent event or is not selected for. The region of homology between the vectors carrying the *lacF* marker and the chromosome of *L. lactis* NZ3000 is less than 0.3 kb. It is has been established that the recombination frequency in *L. lactis* is inversely related to the size of the region of homology and is more than 1,000-fold reduced when the region of homology is reduced from 3 to 0.3 kb (2). Remarkably, many of the vectors were also stable on glucose-containing medium. Most probably this is a consequence of their high copy numbers, which are characteristic of plasmids based on the pSH71 replicon (9, 10). The general applicability of the lactococcal *lacF* complementation system has recently been demonstrated by the construction of a food-grade vector, designated pF1846, based on the pSH71 replicon, which was also stably maintained under lactose selection (17). However, pFI846 still contained the transcriptional terminator of pSH71 and hence needed an additional promoter to drive expression of the *lacF* gene, limiting its application as an expression vector.

The application potentials of the expression vectors pNZ2106 and pNZ2107 have been tested by cloning the promoterless *E. coli gusA* gene under control of the *lacA* promoter and then analyzing its expression by blue-white colony screening of *L. lactis* and determining β-glucuronidase activities in lysates (18). These experiments showed the utility of the *pepN* terminator in pNZ2107, since with this vector the *gusA* gene was readily cloned and expressed, while with pNZ2106, lacking this terminator, only a single blue colony was obtained among 500 transformants. This suggests that most ligation products obtained with pNZ2106 were structurally unstable. Similar structural instability was observed during cloning of the *gusA* gene in the chloramphenicol resistance vector pNZ2103, illustrating the need for evaluating the application potentials of newly constructed vectors. While structural instability during gene cloning in *L. lactis* and other lactic acid bacteria has not been reported frequently (10), it has been observed in several cases, especially when the strong *lacA* promoter was used on high-copy-number plasmids (11, 18, 24). Therefore, this instability was studied in more detail by the characterization of pNZ2118, the plasmid found in the single blue colony obtained during the cloning of the *gusA* gene in pNZ2106. Sequence analysis of the *lacA* promoter region of pNZ2118 showed it to contain a mutation in the *lacA* promoter that involved the first thymidine residue of the TATAAT box (actual position, −11), which was replaced by a cytosine (Fig. 4). Although a systematic mutation analysis of the lactococcal promoter sequences has yet to be reported, this mutation is expected to reduce the promoter activity, since the first thymidine of this canonical −10 region is highly conserved in *L. lactis* promoters (10). It is possible that the mutated *lacA* promoter retains some residual activity that, in conjunction with the high copy number of pNZ2118, is responsible for the low level of *gusA* expression. Alternatively, it is possible that the residual *gusA* expression of pNZ2118 is due to read-through from the *repC* promoter, which also drives expression of the *lacF* gene. The latter possibility would also explain why *L. lactis* NZ3000 harboring pNZ2118 shows constitutive β-glucuronidase production (Table 2). However, footprint studies have shown that the LacR repressor protects the −31 to +6 region of the *lacA* promoter from DNase I digestion (26), and hence the mutation at position −11 may not only affect promoter efficiency but also result

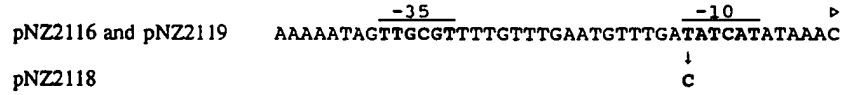

FIG. 4. Promoter region of the *lacA* promoter in pNZ2116, pNZ2119, and pNZ2118. The mutation found at position −11 in the pNZ2118 promoter is indicated, as are the −35 and −10 regions and the transcription initiation start site (arrowhead).

in a reduced binding of the LacR repressor, leading to the observed noninducible phenotype.

The most versatile expression vectors carrying the *lacF* marker that were constructed are the high-copy-number, stable plasmid pNZ2107 and its polylinker-containing derivatives pNZ2122 and pNZ2123, which contain the *lacA* promoter and the *pepN* terminator. When pNZ2107 is equipped with the *gusA* gene, such as in pNZ2119, and introduced in *L. lactis* NZ3000, it specifies a high level of β-glucuronidase activity that is induced twofold by growth on lactose compared with growth on glucose. Under full inducing conditions, pNZ2119 shows a level of β-glucuronidase activity approximately 10-fold-higher than that encoded by a previously constructed plasmid, pNZ276, consisting of pNZ124 carrying the *lacR* gene and *lacA-gusA* promoter fusion (18). This difference can be partly explained by the absence in pNZ2107 of the *lacR* repressor gene, which when present, such as in pNZ276, is known to reduce the efficiency of the *lacA* promoter but to increase the repression of this promoter in multicopy plasmids by preventing titration of the chromosomally encoded LacR repressor (24, 25). However, it cannot be excluded that the high and twofold-controlled *gusA* expression level is due to additional transcriptional read-through from the *repC* promoter and possibly to stabilization of the transcript as a result of the presence of the *pepN* terminator.

In this work we have described the construction and evaluation of a stable and convenient lactococcal food-grade cloning and expression system based on *lacF* complementation. Several of these vectors have been used for the cloning and overexpression of lactococcal genes in *L. lactis* (10, 11). The nucleotide sequences of all of the developed vectors are known, and they consist entirely of *L. lactis* DNA, indicating that they can be used for the improvement of lactococci by self-cloning, the simplest form of genetic modification that employs only homologous DNA and has a distinct regulatory status (10). This will allow for the further development of lactococci as acceptable hosts for the production of proteins, peptides, or metabolites for the food industry.

ACKNOWLEDGMENTS

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Isolation of *Lactococcus lactis* nonsense suppressors and construction of a food-grade cloning vector

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Summary

Nonsense suppressor strains of *Lactococcus lactis* were isolated using plasmids containing nonsense mutations or as revertants of a nonsense auxotrophic mutant. The nonsense suppressor gene was cloned from two suppressor strains and the DNA sequence determined. One suppressor is an ochre suppressor with an altered $tRNA_{Gln}$ and the other an amber suppressor with an altered $tRNA_{Ser}$. The nonsense suppressors allowed isolation of nonsense mutants of a lytic bacteriophage and suppressible auxotrophic mutants of *L. lactis* MG1363. A food-grade cloning vector based totally on DNA from *Lactococcus* and a synthetic polylinker with 11 unique restriction sites was constructed using the ochre suppressor as a selectable marker. Selection, following electroporation of a suppressible purine auxotroph, can be done on purine-free medium. The *pepN* gene from *L. lactis* Wg2 was subcloned resulting in a food-grade plasmid giving a four- to fivefold increase in lysine aminopeptidase activity.

Introduction

Lactococcus lactis is an important industrial organism used in dairy fermentations to produce many cheese varieties and cultured milk products such as buttermilk. Genetic modification of *Lactococcus* is desirable in order to improve, among other properties, the bacteriophage resistance, acid production and production of flavour compounds by industrially important strains. As live *Lactococcus* is present in many of the resulting food products, it is essential that the technology used to modify strains results in food-grade microorganisms. Introduction of *Lactococcus* DNA or short synthetic sequences into a *Lactococcus* cell should not change the food-grade status of the resulting strain.

Nonsense mutations arise upon alteration of a codon, in a protein coding sequence, to one of the nonsense codons. Nonsense mutations lead to premature translation termination and result in the production of truncated proteins which are usually inactive. Mutation of a tRNA gene can result in an altered tRNA capable of recognizing a nonsense codon as a sense codon, thus suppressing nonsense mutations. Amber suppressors suppress only amber (TAG) mutations while ochre suppressors suppress both amber and ochre (TAA) mutations. The phenotype of a nonsense mutant is conditional, i.e. it is dependent upon whether or not the strain contains a suppressor. Nonsense mutants and nonsense suppressor strains have played an essential role in the rapid development of the genetics of bacteria and bacteriophages.

Here we describe the isolation of *Lactococcus* nonsense suppressor strains using two plasmids containing nonsense mutations, the cloning and characterization of the nonsense suppressor genes, and the isolation of suppressible mutations in the purine biosynthetic pathway and in a bacteriophage attacking *Lactococcus*.

We also describe the construction of a cloning vector consisting exclusively of DNA sequences from *Lactococcus* and short regions of synthetic DNA. The vector contains the *L. lactis* subsp. *lactis* biovar diacetylactis citrate plasmid minimal replicon (Pedersen *et al.*, 1994), a cloned *Lactococcus* nonsense suppressor gene, and a synthetic polylinker and has a total size of 2 kb. The nonsense suppressor is a selectable marker when used in combination with a strain containing a nonsense mutation in the purine biosynthetic pathway. This vector allows the construction of food-grade strains containing an increased copy number of desirable *Lactococcus* genes and its use is illustrated by the cloning and overexpression of the *Lactococcus pepN* gene encoding lysine aminopeptidase.

Results

Isolation of nonsense suppressor strains

Plasmid pFDi10, containing the ery_{am} and cat_{am} alleles (Fig. 1), was introduced into *L. lactis* strain MG1363 by electroporation to produce strain FD73. Colonies were tetracycline resistant (Tet^R), chloramphenicol sensitive (Cam^S) and erythromycin sensitive (Ery^S), indicating that

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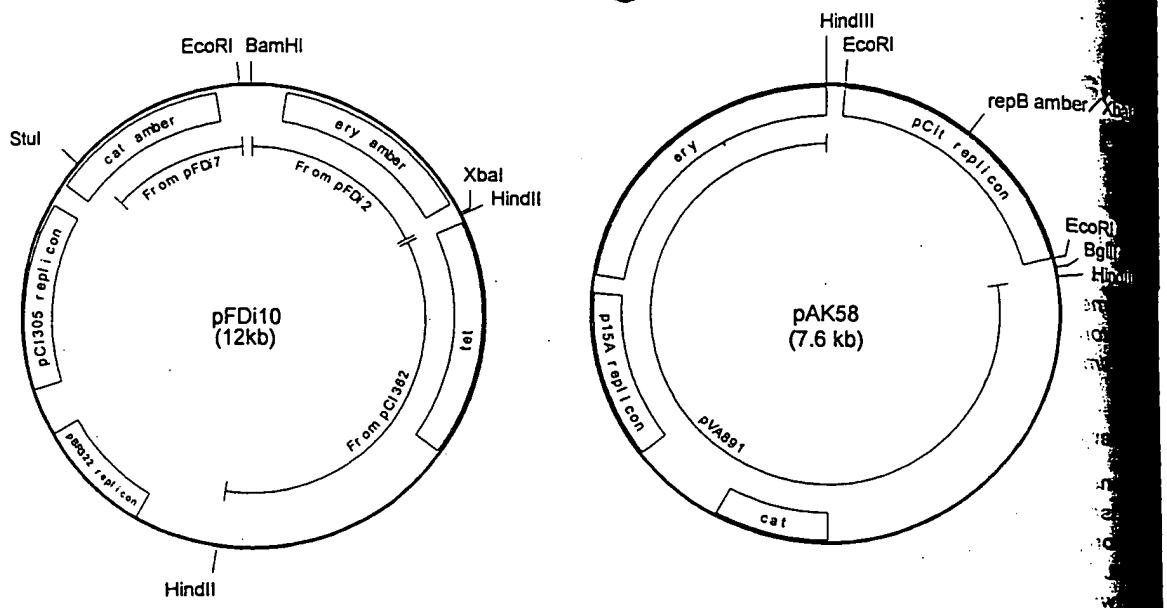

Fig. 1. Plasmids pFDi10 and pAK58 are shown. The following designations are used: cat, ery, and tet indicate resistance to chloramphenicol, erythromycin and tetracycline, respectively; amber indicates the presence of a suppressible nonsense mutation. The construction of pFDi10 is described in the text and the construction of pAK58 is described by Pedersen *et al.* (1994).

MG1363 cannot suppress the amber mutations. FD73 was mutagenized with ethyl methane sulphonate (EMS) and colonies resistant to erythromycin and chloramphenicol were selected. Ten independent mutants were purified and one of these, FD87, was chosen for further analysis.

Extraction of pFDi10 from FD87 and transformation into R594 produced Tet^R Ery^S Cam^S colonies, indicating that the nonsense mutations had not reverted. Curing of pFDi10 from FD87 produced FD100 which is sensitive to tetracycline, chloramphenicol and erythromycin, showing that FD87 is not a mutant with multiple drug resistance. Reintroduction of pFDi10 into FD100 resulted in resistance to all three antibiotics. Thus, FD100 has all of the properties expected of a nonsense suppressor strain.

Plasmid pAK58 (Fig. 1) contains the replication region of the citrate plasmid of *L. lactis* subsp. *lactis* biovar diacetylactis engineered to contain an amber mutation in the *repB* gene and cloned in pVA891. It was constructed using the polymerase chain reaction with primers with specific mismatches and contains an amber mutation (TAG) in the *repB* gene with the concomitant introduction of an *Xba*I restriction site (TCTAGA). This plasmid replicates in FD100 but not in MG1363 (Pedersen *et al.*, 1994).

Electroporation of MG1363 with pAK58 yields erythromycin-resistant (Ery^R) colonies at 10^{-5} the frequency obtained on electroporation with an identical plasmid lacking the amber mutation. Analysis of rare MG1363 transformants revealed two which contain an intact pAK58. The *Xba*I site is still present, indicating that the $repB_{am}$ mutation has not reverted. Curing of pAK58 from one of these, designated NJ1(pAK58), produced strain NJ1 which is Ery^S and can be transformed by pAK58 at a high frequency. Transformation of NJ1 by pFDi10 produced Tet^R colonies at a high frequency but these were found to be Ery^S. Thus NJ1 contains a nonsense suppressor with specificity that differs from that of FD100.

Cloning and sequencing of nonsense suppressor gene

From FD100. DNA fragments containing the suppressor gene may be cloned by their ability to suppress the nonsense mutation in the ery_{am} gene. Plasmid pFDi3 is a shuttle vector made by cloning the ery_{am} gene from pFDi2 into pCI372 as a 2.2 kb *Xba*I–*Bam*HI fragment. Chromosomal DNA from FD100 was digested with *Hin*dIII, cloned into *Hin*dIII-digested pFDi3 and electroporated into MG1363 selecting erythromycin-resistant (Ery^R) transformants. Analysis of plasmid DNA from 10 Ery^R transformants revealed that all have a 3.2 kb *Hin*dIII fragment in common. Plasmid pFDi12 contains only this fragment. Deletion of a 1.0 kb *Hin*dII fragment from pFDi12 produced pFDi14, which contains the suppressor gene, as it is able to suppress the nonsense mutation in the phage MPC100a12 (see below). Deletions of pFDi12 were made with exonuclease III and deletion-containing transformants were tested for suppressor activity with MPC100a12. The smallest plasmid with suppressor

activity (pFDi18) and the largest plasmid without suppressor activity (pFDi19) were saved for DNA sequencing.

The DNA sequence of both strands of the insert in pFDi18, pFDi19 and part of the insert in pFDi14 was determined and the relevant sequence is presented in Fig. 2. Comparison with sequences in the DNA sequence databank revealed homology to $tRNA_{gln}$ from a variety of organisms. At the position of the anticodon, the FD100 suppressor $tRNA_{gln}$ has the triplet 3′-ATT-5′ capable of pairing with the codons TAA (ochre) and TAG (amber). Thus this suppressor is an ochre suppressor.

Sequencing of the wild-type allele from MG1363 revealed that this gene is indeed a $tRNA_{gln}$ gene, with the anticodon 3′-GTT-5′. We suggest the designation *supB* for this ochre suppressor and the designation *glnU* for the corresponding $tRNA_{gln}$ gene. Sequencing of this $tRNA_{gln}$ gene from 11 other *L. lactis* nonsense suppressor mutants revealed that nine had the wild-type anticodon and two had the same ochre suppressor anticodon.

From NJ1. A DNA fragment of NJ1 containing the suppressor gene will allow pAK58 to replicate in MG1363. Chromosomal DNA from NJ1 was partially digested with *Sau*3AI and cloned into the *Bgl*II site of pAK58. MG1363 was electroporated with ligation mixes and Ery^R colonies selected. One such colony contained a plasmid designated pAK85 containing an insert of 5.1 kb. *Xba*I fragments from pAK85 were subcloned in pCI372 and tested for suppression of the amber mutation in pAK58 by electroporation of MG1363 with a mixture of the pCI372 derivative (chloramphenicol-resistant; Cam^R) to be tested and pAK58 (Ery^R). Since the *repB* gene of pCI372 cannot complement the amber mutation in the *repB* gene of pAK58 (Pedersen *et al.*, 1994), Ery^R Cam^R colonies will only be obtained if the pCI372 derivative contains the nonsense suppressor allowing replication of pAK58. One clone, pAK89, contains a 2.8 kb *Xba*I fragment from pAK85 and has suppressor activity by this criterion. Deletion of a 1.7 kb *Eco*RI fragment from pAK89 produced pAK89.1 which also retains suppressor activity.

The DNA sequence of the 1.1 kb insert in pAK89.1 was determined on both strands and the relevant sequence is presented in Fig. 3. This fragment codes for a tRNA with the anticodon 3′-ATC-5′, capable of pairing only with amber codons. Thus NJ1 is an amber suppressor. Homology searches revealed that this suppressor was probably a mutant $tRNA_{ser}$. This was confirmed by sequencing of the wild-type allele, which revealed the anticodon 3′-AGC-5′ recognizing the serine codon 5′-TCG-3′. The designation *supD* is suggested for this amber suppressor and *serU* for the corresponding $tRNA_{ser}$ gene. Sequencing of this $tRNA_{ser}$ gene from 11 other nonsense suppressor mutants revealed that all had the wild-type anticodon. Thus our mutant collection contains at least one additional suppressor.

Upstream of the *serU* gene is the beginning of a gene transcribed in the opposite direction. This gene contains two ARG box sequences (Glansdorff, 1987) overlapping a consensus promoter, a ribosome-binding site, and an open reading frame coding for a protein with nearly 50% amino acid identity to argininosuccinate synthase (*argG*) from a variety of organisms (our unpublished results).

Isolation of nonsense mutants of bacteriophage MPC100

Dilutions of a mutagenized phage stock were plated on the nonsense suppressor strain FD100. Plaques were picked

```
      10                  30                  50
      .                   .                   .
AATTGCGACAGTGTCTTCATTTGAGGCTGCTTTAGAAGAAGCAATCAAGGAATATAATCT
        Ochre-3 --->
      70                  90                  110
      .                   .                   .          -44
ATCTATTTAAAGAGATTATAAAAAATTATTGATATTTCTTTGAAATAAATAAGTTAAAAC
                                                %%%%
pFDi18    pFDi19      150                 170
|         |
-35               -10 .                   .                   .
TTGAAATTTATGAGGGTTTTTGGTAAAATATTTCTTGTCGTCATCAAGCGATCTTGGGGT
******  Ochre-2 --->  ######                                  supB
      190                 210                 230
      .                   .                   .
ATAGCCAAGCGGTAAGGCAAGGGACTTTAACTCCCTCATGCGTTGGTTCGAATCCAGCTA
                          &&&
      250                 270                 290
      .                   .                   .
CCCCAGTAAAAAAACTTTAAAGGAAACGTTGTTTCCTTTTTTCTTTTTACTAAAATATGA
                  -------->   <---------
      310                 330
      .                   .
TAGAATAGGGGAAAGCATTTATGAAAATGT
      <--- Ochre-1
```

Fig. 2. DNA sequence of the ochre suppressor allele, *supB*, isolated from FD100. A potential promoter is indicated (%, −44 region; *, −35 region; #, −10 region). The arrows indicate an inverted repeat forming part of a potential transcription terminator. The underlined bases are expected to form an active tRNA after transcription and post-transcriptional modification. The anticodon is indicated (&). The start of *Lactococcus* DNA in the plasmids pFDi18 (nucleotide 121) and pFDi19 (nucleotide 132) is indicated. The primers used for PCR amplification and DNA sequencing are indicated by double underlining. This sequence appears in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Library under the Accession Number L35277. Position 209 is a G in *glnU*.

Table 1. PCR primers.

Primer	Sequence
Ochre-1	CGAATTCATAAATGCTTTCCCCTATTC
Ochre-2	CGAATTCTTGAAATTTATGAGGGTTTTTGG
Ochre-3	CGAATTCGACAGTGTCTTCATTTGAGGC
Amber-1	CCACCTGAATATGCCAAGAC
Amber-2	CGAATTCAACATTTTTGTATAAATATGCG
Amber-3	CGAATTCATATTTGATTAATGAGAATATGGAACC

The *supB* allele is a suitable selectable marker when combined with the purine auxotroph DN209. This strain only grows in purine-free medium in the presence of the ochre suppressor. DNA of pAK95 and pKR41 was digested with *Eco*RI, mixed, ligated and electroporated into DN209 selecting prototrophs. This selection ensures that colonies contain plasmids with the suppressor gene and the citrate plasmid replicon. Some plasmids will also contain pIC19H. These were obtained by pooling several hundred colonies, extracting plasmid DNA and transforming *E. coli* selecting Amp^R. One plasmid with the desired structure was designated pAK102.

All of pIC19H except the polylinker was deleted by digesting pAK102 with *Hin*dIII, self ligating, and electroporating DN209 selecting prototrophs. The resulting colonies contained a single plasmid of 2.0 kb containing the citrate plasmid replicon, the ochre suppressor and the polylinker. One was saved and the plasmid designated pFG1. The structure pFG1 is illustrated in Fig. 4. The resulting polylinker is identical to that found in pIC19R (Marsh *et al.*, 1984) and contains the following unique sites: *Sma*I, *Bam*HI, *Sal*I, *Pst*I, *Hin*dIII, *Nru*I, *Xho*I, *Sac*I, *Bgl*II, *Xba*I and *Eco*RV.

Electroporation of DN209 with pFG1 is efficient (>10^6 prototrophic transformants per μg of DNA). The resulting transformants grow in DN medium as well as in milk supplemented with glucose and casamino acids. The plasmid copy number was determined to be 5–9 copies per chromosome equivalent. Plasmid loss is uncommon under the selective conditions in milk (0.2% plasmid-free derivatives after 40 generations). Because of the expected stability of the suppressor tRNA molecules, some growth may occur following plasmid loss, even under selective conditions. In the non-selective medium GM17, plasmid-free derivatives comprise 3.4% of a culture after 40 generations.

Cloning of the L. lactis pepN *gene into pFG1*

The lysine aminopeptidase gene of *L. lactis* strain Wg2 has been cloned, producing a plasmid called pSTO5 (Strøman, 1992). This gene was originally named *lap*, but was renamed *pepN* because of its near identity to the partial sequence of the MG1363 *pepN* gene published by van Alen-Boerrigter *et al.* (1991). Plasmid pSTO3 is a derivative of pSTO5 containing the entire Wg2 *pepN* gene from which upstream and downstream regions not essential for *pepN* expression have been removed *in vitro* (P. Strøman, personal communication). A 3.5 kb *Bam*HI–*Sac*I fragment was subcloned from pSTO3 into pFG1. DN209 was electroporated with the ligation mix and prototrophic colonies selected on DN medium. Plasmid minipreps were used to find clones with the desired insert. One such plasmid is pFG2, which has a copy number of 5–9. DN209 (pFG2) produces 230 units of lysine aminopeptidase per mg of protein, while DN209 (pFG1) produces 49 units per mg protein.

Discussion

tRNA genes

We have determined the DNA sequence of two tRNA genes, *glnU* and *serU*, from *L. lactis* MG1363. Both genes contain a single tRNA species preceded by a potential promoter and followed by a potential transcription terminator. Since pFDi18 has suppressor activity and pFDi19 does not, the *glnU* promoter indicated must be functional in MG1363. The promoters of both genes contain the −44 region postulated to precede genes with high levels of expression or showing growth-rate-dependent regulation (Nilsson and Johansen, 1994). Previously sequenced tRNA genes from *Lactococcus* have

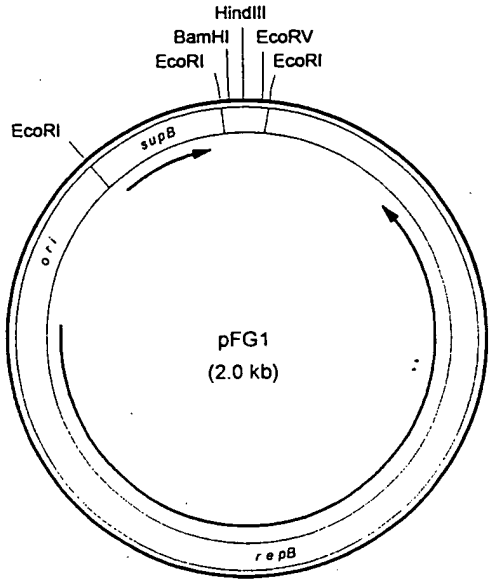

Fig. 4. The food-grade vector, pFG1. The various components and the construction of pFG1 are described in the text. Arrows indicate the direction of transcription of *supB* and *repB*. Restriction sites used during the construction and selected sites in the polylinker are indicated.

been either part of a rRNA operon (Chiaruttini and Milet, 1993) or part of an operon with several tRNA species (Nilsson and Johansen, 1994). The sequences downstream of the *glnU* and *serU* genes do not contain additional tRNA genes.

The tRNA encoded by these genes can be folded into typical 'cloverleaf' structures (not shown) and contain the invariant bases typical of tRNA (Rich, 1977). The serine tRNA contains a large variable loop, often seen in $tRNA_{ser}$. Neither gene contains the 3'-terminal sequence CCA, so this must be added post-transcriptionally.

The mutational event leading to the amber suppressor was a spontaneous G to T transversion while the ochre suppressor arose in an EMS mutagenized culture by a G to A transition. Both mutations change the anticodon producing typical nonsense-suppressing tRNA. No other changes were detected in this region of the chromosome in these mutants.

Eight nonsense-suppressing mutants without alterations in the *glnU* or *serU* genes have also been isolated. These are good candidates for the cloning of additional *Lactococcus* tRNA genes.

Nonsense mutants and nonsense suppressors

We have described three methods for the isolation of nonsense suppressing *Lactococcus* strains, and nonsense mutations in four genetic systems. Nonsense mutations can be isolated in *Escherichia coli* using selection techniques involving *E. coli* nonsense suppressor strains, constructed *in vitro* using PCR with mismatch primers, found by screening *Lactococcus* auxotrophs with cloned suppressor genes or by screening mutagenized phage stocks for the ability to grow only on suppressor strains. *Lactococcus* nonsense suppressors can be isolated by selecting mutants able to suppress nonsense mutations in antibiotic-resistance genes, in plasmid replication genes or as revertants of nonsense auxotrophic mutants.

The system of nonsense suppressors and nonsense mutants described here is a valuable tool for the genetic analysis of *Lactococcus* and their plasmids and bacteriophages. The amber mutant of the citrate plasmid replicon helped demonstrate that translation of the *repB* gene is essential for plasmid replication (Pedersen *et al.*, 1994). Genetic analysis of bacteriophage and bacteriophage–host interactions in other organisms is greatly facilitated by analysis of nonsense mutants. Likewise, nonsense mutants have proved to be valuable for studying the effect of various genes on the metabolic properties of the cell.

Cloning vectors

Plasmids with nonsense mutations in the replication region will be effectively contained, for example in the mixed cultures often used in dairy fermentations. Plasmid[illegible] fer to a non-suppressing host will result in a failure to [illegible] cate in the new host.

In addition to their previously discussed uses, no[illegible] suppressors can be used as small selectable [illegible] (Huang *et al.*, 1988). We have used the cloned ochr[illegible] pressor, *supB*, combined with a purine auxotroph to [illegible] struct a food-grade selection system and cloning [illegible] for *Lactococcus*. This system allows the genetic mo[illegible] tion of *Lactococcus* without the introduction of DNA [illegible] other species and the resulting strains should retai[illegible] food-grade status.

Nonsense suppressors have several advantages [illegible] other food-grade selectable markers, usually [illegible] drate fermentation genes or genes conferring resis[illegible] to bactericidal agents. They are small, typically contain[illegible] less than 300 bp, and so integration of the plasmid [illegible] chromosome, by homologous recombination, will [illegible] mediated by the suppressor gene (Biswas *et al.*, [illegible] Their gene product is RNA rather than protein and [illegible] contain no enzymatic or antibacterial activity. Their [illegible] much more flexible, allowing selection to be main[illegible] in a variety of media. The described purine auxotro[illegible] allow selection for plasmid maintenance in milk, the [illegible] ium of choice for cheese making. Introduction of a [illegible] sense mutation in an essential gene will allow sele[illegible] in all media.

The food-grade vector pFG1 has several useful prop[illegible] ties. It is small and contains a versatile polylinker. [illegible] citrate plasmid replicon replicates by theta replica[illegible] (Pedersen *et al.*, 1994), which is expected to give [illegible] increased structural stability (Kiewiet *et al.*, 1993) [illegible] limited host range seen for other theta-replicating *Lac*[illegible] *coccus* plasmids (e.g. Lucey *et al.*, 1993). The copy [illegible] ber of pFG1 has been determined to be 5–9 copies [illegible] genome equivalent.

Lactococcus strains contain a broad spectrum of ami[illegible] peptidases, the activities of which play a significant role [illegible] the development of flavour in cheese. We have develop[illegible] a pair of isogenic strains (DN209 (pFG1) and DN20[illegible] (pFG2)) differing four- to fivefold in the level of the pep[illegible] gene product. Van Alen-Boerrigter *et al.* (1991) over[illegible] expressed *pepN* 20-fold by means of cloning on a plasm[illegible] with a copy number of approximately 35. The difference [illegible] the levels of overexpression probably reflects the differ[illegible] ence in the copy number of the two vector systems used. Because the construction of van Alen-Boerrigter *et* [illegible] (1991) was not food-grade, the effect of overexpression on flavour development could not be assessed. In contrast, DN209 (pFG1) and DN209 (pFG2) were constructed using only synthetic sequences and DNA from *Lactococcus*, and should retain their food-grade status. These strains will allow an assessment of the effect of overexpressing lysi[illegible] aminopeptidase on flavour development in cheese.

Table 2. Bacterial strains.

	Description	Source/Reference
IL1363	Plasmid-free derivative of NCDO 712	Gasson (1983)
	MG1363(pFDi10)	This work
	Ochre-suppressor mutant of MG1363	This work
	Amber-suppressor mutant of MG1363	This work
	Purine auxotroph of MG1363	This work
	supE44 lacΔU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 Φ80lacΔZM15	Hanahan (1983)
	sup° mutD5 rpsL azi galU95	Silhavy *et al.* (1984)
	sup° rpsL179 galK2 galT22 lac-3350	Campbell (1965)
BR2024	*sup° ara argE Δ(lac-pro) nalA rpoB thi recA56 srl*::Tn*10*	Austin and Wierzhicki (1983)
BR2025	*supD* mutant of BR2024	Austin and Wierzhicki (1983)
BR2026	*supE* mutant of BR2024	Austin and Wierzhicki (1983)
BR2027	*supF* mutant of BR2024	Austin and Wierzhicki (1983)

Experimental procedures

Bacterial strains, bacteriophage, plasmids and media

Strains of *L. lactis* and *E. coli* are listed in Table 2. Bacteriophage MPC100 is a prolate-headed phage isolated from a dairy whey sample (our unpublished results). Plasmids are listed in Table 3.

E. coli was grown at 37°C using LB medium (Miller, 1972) supplemented, when necessary, with antibiotics at the following concentrations: ampicillin, 50 mg l^{-1}; erythromycin, 250 mg l^{-1}; chloramphenicol, 25 mg l^{-1}; tetracycline, 10 mg l^{-1}. AB minimal medium (Clark and Maaløe, 1967) was used for LE30.

L. lactis was grown at 30°C using M17 (Terzaghi and Sandine, 1975) containing 0.5% glucose instead of lactose as carbon source. Antibiotics were used at the following concentrations: erythromycin, 1 mg l^{-1}; chloramphenicol, 5 mg l^{-1}; tetracycline, 10 mg l^{-1}. For work with bacteriophages, plates and broth contained 5 mM $CaCl_2$ and 5 mM $MgSO_4$.

A purine-free medium (DN medium) for *L. lactis* was made by adding glucose (5 g l^{-1}), vitamin-free casamino acids (5 g l^{-1}), vitamins, sodium acetate (2 g l^{-1}) and asparagine (80 mg l^{-1}) to AB minimal medium (Clark and Maaløe, 1967) in which NaCl was reduced from 3 g l^{-1} to 1 g l^{-1}. Casamino acids were boiled with activated charcoal to remove residual purines, filtered, neutralized and filter-sterilized. The final concentration of the added vitamins was 0.1 mg l^{-1} for biotin, 1 mg l^{-1} for folic acid, riboflavin, nicotinic acid, thiamine, and pantothenic acid, and 2 mg l^{-1} for pyridoxal.

Plasmid curing was performed by growing cells under non-selective conditions for 40 generations, plating on non-selective plates, and then replica plating on selective and non-selective plates. Typically, 10% of colonies had lost the selective marker. Plasmid loss was confirmed by plasmid analysis as described below.

DNA isolation and manipulations

Digestion with restriction enzymes, ligation, plasmid preparations from *E. coli* and transformation of *E. coli* were essentially as described by Sambrook *et al.* (1989). Plasmid DNA for DNA sequencing and electroporations was extracted from *E. coli* using the Qiagen plasmid kit (Diagen).

Chromosomal DNA was extracted from *L. lactis* as described by Johansen and Kibenich (1992); plasmid DNA was as described by Pedersen *et al.* (1994). Plasmids were introduced into *Lactococcus* by electroporation of glycine-grown competent cells (Holo and Nes, 1989). Ligation mixes were ethanol-precipitated and resuspended in H_2O before electroporation.

Deletions of plasmid pFDi14 were made using the Erase-a-Base kit (Promega Inc.) according to the manufacturer's instructions. An exonuclease III-resistant 3′ overhang was made with *Sac*I while *Bam*HI was used to give an exonuclease III-sensitive 5′ overhang. Thus, deletions extend

Table 3. Plasmids.

Plasmid	Properties	Replicon(s)/ Parent plasmid	Source/ Reference
pVA891	Cam^R Ery^R	pACYC184	Macrina *et al.* (1983)
pCI160	Tet^R Amp^R	pBR322	Hill *et al.* (1988)
pCI372	Cam^R	pBR322 and pCI305	Hayes *et al.* (1990)
pCI3340	Cam^R	pBR322 and pCI305	Hayes *et al.* (1990)
pIC19H	Amp^R	pBR322	Marsh *et al.* (1984)
pFDi2	Cam^R Ery_{am}[a]	pVA891	This work
pFDi3	Cam^R Ery_{am}	pCI372	This work
pFDi6	Cam^R Ery^R	pCI3340	This work
pFDi7	Cam_{am} Ery^R	pFDi6	This work
pFDi8	Tet^R Cam^R	pCI372	This work
pFDi9	Tet^R Cam_{am}	pFDi8	This work
pFDi10	Tet^R Cam_{am} Ery_{am}	pFDi9	This work
pFDi12	Cam^R Ery_{am} *supB*	pFDi3	This work
pFDi14	Cam^R *supB*	pFDi12	This work
pFDi18	Cam^R *supB*	pFDi14	This work
pFDi19	Cam^R *supB*$^-$	pFDi14	This work
pAK58	Ery^R pCit-*repB*$_{am}$	pVA891 and pCT1138	Pedersen *et al.* (1994)
pAK85	Ery^R pCit-*repB*$_{am}$ *supD*	pAK58	This work
pAK89	Cam^R *supD*	pCI372	This work
pAK89.1	Cam^R *supD*	pAK89	This work
pKR41	Amp^R pCit-*rep*$^+$	pIC19H and pCT1138	Pedersen *et al.* (1994)
pAK95	Amp^R *supB*	pIC19H	This work
pAK102	Amp^R *supB* pCit-*rep*$^+$	pKR41 and pAK95	This work
pFG1	*supB* pCit-*rep*$^+$	pAK102	This work
pSTO3	Amp^R *pepN*	pSTO5	P. Strøman
pFG2	*supB* pCit-*rep*$^+$ *pepN*	pFG1	This work

a. *am*, amber.

from the pCI372 polylinker into the inserted DNA fragment. Incubation was at 30°C and samples were removed at 1 min intervals for 15 min. Self-ligated plasmids were electroporated into MG1363, selecting for Cam^R.

DNA sequencing

The nucleotide sequence of both strands of cloned *Lactococcus* DNA was determined using the dideoxy chain termination method (Sanger *et al.*, 1977). For sequencing of clones inserted in pCI372, primer 'pBR322 Pst CW' (Promega Inc.) and the custom primer 'pCI3340-1' (sequence CCTTTA-CCTTGTCTACAAACC) were used to sequence from the vector into the insert. Additional custom primers were designed based on the sequence data obtained and synthesized on an Applied Biosystem 380A synthesizer using the recommended protocol.

Sequencing of chromosomal genes without cloning was done following specific amplification of the region to be sequenced by PCR, using conditions described by Pedersen *et al.* (1994). For the *glnU* region, amplification was with primers Ochre-1 and Ochre-3 (Table 1 and Fig. 2) and, for the *serU* region, Amber-1 and Amber-3 (Table 1 and Fig. 3). The fragments obtained were purified by extraction with phenol/chloroform/isoamyl alcohol (24:24:1) followed by ethanol precipitation. The fragments were sequenced with end-labelled, internal primers (Ochre-2 and Amber-2, respectively) using the dsDNA Cycle Sequencing System (BRL Life Technologies, Inc.), allowing sequencing of 250–300 bp.

Computer analysis of the DNA sequences was with the GCG software package Version 7.1 (UNIX) (Devereux *et al.*, 1984) and EMBL DNA sequence database release 34.0.

Mutagenesis of bacteria, bacteriophages and plasmids

Mutagenesis of MG1363 with EMS was performed as described by Nilsson and Lauridsen (1992). Samples were removed at various times to give pools of mutagenized cells.

A phage stock of MPC100 with a titre of 2×10^{10} PFU ml^{-1} was mutagenized for 22 h with hydroxylamine as described in Silhavy *et al.* (1984). The survival rate was 3×10^{-3}.

Mutagenesis of plasmids was done using the mutator strain LE30. Cells were grown in AB minimal medium, made competent, and transformed with the plasmid to be mutagenized. Selection was for the marker in which mutations were not sought. Plasmids were extracted from pools of over 5000 colonies and used as a mutagenized plasmid stock.

Isolation of nonsense mutations in antibiotic-resistance genes

E. coli strain R594 was transformed with a mutagenized stock of plasmid pVA891 and Cam^R transformants were selected. Over 5000 colonies were pooled and enriched for Ery^S colonies by ampicillin enrichment (Miller, 1972). Out of 300 Cam^R colonies tested, 113 Ery^S derivatives were found. These were pooled and plasmids extracted and used to transform the *E. coli* suppressor strains BR2025 and BR2026 and the suppressor-free *E. coli* strain, BR2024, selecting for Ery^R or Cam^R. Cam^R transformants were obtained with all three strains while Ery^R transformants were only obtained with BR2026. A plasmid designated pFDi2 was isolated from one of the Ery^R Cam^R BR2026 transformants. Transformation of BR2024, BR2025 and BR2026 with pFDi2 proved the presence of a nonsense mutation in the erythromycin-resistance gene. This mutation is efficiently suppressed by *supE* and weakly suppressed by *supD* and is therefore designated amber.

The shuttle plasmid pFDi6 was constructed by inserting the Ery^R gene of pVA891 into pCI3340 as a 1.7 kb *Hin*dIII fragment. A derivative of pFDi6, designated pFDi7, carrying a nonsense mutation in the Cam^R gene was isolated by enriching for Cam^S transformants by a method analogous to that described above. The cat_{am} allele in pFDi7 was suppressed by *supD*, *supE*, and *supF*.

Construction of pFDi10

A plasmid containing the ery_{am} and cat_{am} alleles was constructed as follows: the Tet^R gene of Tn*919* was cloned from pCI160 into pCI372 as a 4.2 kb *Hin*dII fragment to produce plasmid pFDi8; the Cam^R gene of pFDi8 was replaced by the cat_{am} allele by substituting a 1.5 kb *Eco*RI–*Stu*I fragment from pFDi7 for that in pFDi8, producing plasmid pFDi9. The ery_{am} gene from pFDi2 was inserted into pFDi9 as a *Xba*I–*Bam*HI fragment to produce pFDi10. The presence of the two amber alleles in pFDi10 was confirmed by transformation of R594 and BR2026 and testing on the various antibiotics. The structure of pFDi10 is illustrated in Fig. 1.

Plasmid copy-number determination

Total genomic DNA from fresh saturated cultures in DN medium of DN209, DN209 (pFG1) and DN209 (pFG2) was digested with *Hin*dIII, separated by agarose gel electrophoresis and transfered to a GeneScreen Plus nylon filter (E. I. du Pont de Nemours) according to standard procedures (Sambrook *et al.*, 1989). The 208 bp PCR fragment produced with the primers Ochre-1 and Ochre-2 was denatured, end-labelled with [γ-^{32}P]-ATP and T4 polynucleotide kinase, and used as a hybridization probe. Hybridization at 42°C in the presence of 50% formamide and washing was by the preferred method of the filter manufacturer. Quantification of ^{32}P in each band was with a Packard Instant Imager (Packard Instrument Co.). The copy number of the plasmid was calculated as the ratio of counts per minute in the plasmid band to counts per minute in the 3.2 kb chromosomal band.

Assay of pepN *expression*

Cultures were grown to saturation in DN medium. Cell-free extracts were prepared by sonication and *pepN* activity was assayed as described by Strøman (1992) using L-lysine-*p*-nitroanilide. One unit of lysine aminopeptidase will hydrolyse 1 nmol substrate min^{-1}. Protein determinations were done with Protein Assay Kit I (Bio-Rad Laboratories).

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